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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/00	A1	(11) International Publication Number: WO 92/05246
		(43) International Publication Date: 2 April 1992 (02.04.92)

(21) International Application Number: PCT/US91/06837

(22) International Filing Date: 20 September 1991 (20.09.91)

(30) Priority data:
588,017 25 September 1990 (25.09.90) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MEDIUM FOR CULTURE OF MAMMALIAN CELLS

(57) Abstract

The invention provides serum-free media for the culture of mammalian cells comprising a synthetic basal medium designed for mammalian cell culture; about 0.1 to about 10 grams per liter hydrolyzed yeast; about 0.1 to about 5 grams per liter of dextran or albumin; about 2 to about 20 milligrams per liter insulin; 0 to about 100 milligrams per liter of a compound selected from the group consisting of transferrin, ferric fructose, ferrous citrate and ferrous sulfate; and a fatty acid component consisting of oleic acid, linoleic acid and linolenic acid in a ratio of about 0.6: 1: 0.14 milligrams of fatty acid per liter.

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AT	Austria	ES	Spain	MG	Madagascar
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\+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

MEDIUM FOR CULTURE OF MAMMALIAN CELLS

Field of the Invention

The present invention relates to the field of cell culture media. More particularly the invention relates to the field of mammalian cell culture media.

Background of the Invention

Beyond a basal nutrient mixture of salts, sugars, amino acids, and vitamins, cells in vitro have also been found to require for proliferation a supplement of poorly defined biological fluids or extracts. Because of availability and ease of storage, the most commonly used supplement is serum.

The use of serum in cell culture media, however, has several disadvantages. Serum is comparatively expensive. Since serum is not a defined component, different lots of serum may vary in the concentration of compounds present and thus result in unpredictable culture growth. Serum may also be contaminated with viruses or mycoplasms. The protein in serum may complicate the purification of cell products from the culture medium.

In efforts to overcome the disadvantages of serum containing medium, researchers have attempted to provide serum-free media by substituting defined or better characterized components for serum. Unfortunately, the complexity of serum and the differing growth requirements of different types of cells has made it difficult to provide such media. For reviews on serum-free media for mammalian cell culture see Rizzino et al. (1979) "Defined Media and the

Determination of Nutritional and Hormonal Requirements of Mammalian Cells in Culture" Nutrition Reviews 37: 369-378; Barnes and Sato (1980) "Serum-free Cell Culture: a Unifying Approach", Cell 22: 649-655; Barnes and Sato (1980) "Methods for Growth of Cultured Cells in Serum-Free Medium", Analyt. Biochem. 102: 255-270; and Bodeker et al. (1985) "A Screening Method To Develop Serum-Free Culture Media For Adherent Cell Lines", Develop. Biol. Standard. 60: 93-100.

U.S. Patent 4,786,599 issued November 22, 1988 to Chessebeuf and Padieu discloses a serum-free animal tissue culture medium containing a mixture of six fatty acids and albumin or dextran. The medium is particularly adapted for the primary culture of rat liver epithelial cells and possibly in the presence of hormones and/or growth factors, for obtaining cell lines, in particular myeloma and hybridoma cell lines.

Media for the serum-free culture of Chinese hamster ovary cells (CHO) have been reported. Gasser et al (1985) In Vitro Cellular & Developmental Biology 21: 588-592 discloses a serum-free medium for the culture of CHO cells. The serum-free medium is composed of a 1:1 mixture of Ham's F12 and modified Eagle's minimum essential media supplemented with transferrin, insulin, and selenium. Mendiaz et al. (1986) In Vitro Cellular & Developmental Biology 22: 66-74 discloses a serum-free medium for the culture of CHO cells composed of a basal medium supplemented with insulin, and ferric sulfate or transferrin, selenium, trace elements, calcium chloride, glutamine, linoleic acid, non-essential amino acids, and insulin.

Pietrzkowski et al (1988) Folia Histochemica et Cytobiologica 26: 123-132 report a serum-free medium for the culture of chick embryo cells containing dextran. Pietrzkowski and Korohoda (1988) Folia Histochemica et Cytobiologica 26: 143-154 report a serum-free medium containing dextran for the culture of chick embryo fibroblasts. In these two publications, the dextran was added to the medium to enhance cell attachment and spreading.

Ohmori (1988) Journal of Immunological Methods 112: 227-233 reports a serum-free medium which is able to support primary antibody responses by cultured murine lymphocytes. This medium is based on a basal medium supplemented with ß-cyclodextrin, insulin, transferrin, albumin, low density lipoprotein, putrescine and alanine.

It is an object of the invention to provide serum-free media for the culture of mammalian cells. It is also object of the invention to provide serum-free media for the culture of mammalian cells transformed to produce recombinant products that increase product yield. It is yet another object of the invention to provide serum-free media for the culture of CHO cells.

Summary of the Invention

The present invention provides media for the culture of mammalian cells. The invention is more particularly pointed out in the appended claims and is described in its preferred embodiments in the following description.

Detailed Description of the Invention

The media of the invention are useful for the culture of mammalian cells. The media of the invention have been found to be useful in the culture of Chinese hamster ovary (CHO) cells, and HAK cells, a baby hamster kidney cell line. The media of the invention have been found not suitable for the culture of myeloma cell lines.

Cells may be grown in batch and continuous culture with the serum-free media of the invention. CHO cells grown in the media of the invention reach higher cell density and show increased recombinant product secretion when compared to CHO cells grown in a serum-containing medium.

The cell culture media of the invention are prepared by adding components to a basal medium designed for mammalian cell culture. The media are prepared in accordance with standard procedures for preparing cell culture media.

Suitable basal media include standard mammalian cell culture media such as Ham's medium, Waymouth MB 752/1 medium, Eagle's medium, Williams E medium, 199 medium and derived

media of the types MEM and MEMα and any combinations of these media. Other standard media used for the culture of mammalian cells are also suitable for use in the invention. A preferred basal medium is the basal medium of Example 1. The preferred basal medium supports cell growth and significantly reduces the size of cell clumps in the media during cell culture.

A yeast hydrolysate such as Yeastolate is added to the basal medium in the amount of from about 0.1 to about 10.0 grams per liter, preferably in an amount of about 5 grams per liter.

Albumin or dextran is added to the basal medium in an amount of from about 0.1 to about 5.0 grams per liter. Preferably either bovine serum albumin or dextran having a molecular weight of about 500,000 is added to the basal medium. Bovine serum albumin is preferably added in the amount of from about 0.1 to about 0.5 grams per liter. Dextran having a molecular weight of about 500,000 such as Dextran T500 is preferably added to the basal medium in the amount from about 0.1 to about 1.0 grams per liter.

Insulin is added to the basal medium in the amount of from about 2.0 to about 20 milligrams per milliliter, preferably in the amount of about 10 milligrams per liter.

Transferrin or transferrin substitute is added to the basal medium in the amount of from about 0 to about 100.0 micrograms per milliliter. Transferrin may be substituted in the medium with ferric fructose (from about 1.0 to about 10.0 milligrams per liter), ferric citrate (from about 1.0 to about 100.0 milligrams per liter), or ferrous sulfate (from about 5.0 micromoles to about 200.0 micromoles per liter).

A mixture of the fatty acids oleic, linoleic and linolenic are added to the basal medium in the ratio of oleic 0.6: linoleic 1: linolenic 0.14 milligrams per liter of medium. In preferred embodiments of the invention, keeping this ratio of fatty acids, oleic acid is preferably added to the basal medium in the amount of from about 0.012 to about 0.12 milligrams per liter; linoleic acid is preferably added to the basal medium in the amount of from about 0.2 to about

5.0 milligrams per liter; linolenic acid is added to the medium in the amount of from about 0.028 to about 0.7 milligrams per liter. Cholesterol is added to the basal medium in the amount of from about 0 to about 10.0 milligrams per liter.

In a preferred embodiment of the invention which is described in further detail in Example 2, calcium chloride ($CaCl_2$) (anhydrous) is added to the basal medium in the amount of from about 0 to about 200 milligrams per liter, preferably in the amount of about 66.67 milligrams per liter. Magnesium sulfate ($MgSO_4$) (anhydrous) is added to the basal medium in the amount of from about 0 to about 100.0 milligrams per liter, preferably in the amount of about 24 milligrams per liter.

The pH of the medium is preferably from about 6.8 to about 7.4. The osmolarity of the medium is preferably from about 280 to 360 milliosmoles.

The basal medium may be stored as a powder at 4°C for one year. The complete medium (basal medium with added supplements) in a liquid form may be stored at 4°C for six months.

Preferred embodiments of the invention are described in the following Examples.

Example 1 Preparation of Basal Medium

The components in the basal media are mixed and ball-mill ground to formulate a homogeneous powder. The powdered media is then dispensed into 100L packets and stored at 4°C.

BASAL MEDIUM COMPONENTS: MR1 SERUM-FREE MEDIA

COMPONENTS	milligrams/liter
INORGANIC SALTS/TRACE ELEMENTS	
NaCl	7066.333000
KCL	341.200000
NaH2PO4.H20	93.333000
Na2HPO4	47.347000
MgC12 6H20	4.050000
MgSO4 (anhydrous)	6.510000
CuSO4.5H20	0.000866
Fe(NO3)3.9H20	0.000033
FeSO4.7H20	0.278000
ZnSO4.7H20	0.287700
MnC12.4H20	0.000033
Na2Se03 (anhyd)	0.172900
AMINO ACIDS	
L-Alanine	41.300000
L-Arginine HC1	112.546700
L-Arginine FB	16.666000
L-Asparagine H20	28.336700
L-Aspartic Acid	24.433300
L-Cystine 2HC1	19.116600
L-Cysteine HC1.H2O	45.040000
L-Cysteine FB	13.333300
L-Glutamic Acid	46.566700
L-Glutamine	292.000000
Glycine	35.833300
L-Histidine HC1.H20	20.986700
L-Histidine FB	5.000000
L-Isoleucine	35.480000
L-Leucine	46.833300
L-Lysine HC1	65.486600
L-Methionine	11.493300
L-Phenylalanine	20.653300
L-Proline	34.833300
L-Serine	15.166700
L-Threonine	33.300000
L-Tryptophan	7.346700
L-Tyrosine 2Na2H20	36.776700
L-Valine	35.900000
VITAMINS/MISC. COMPONENTS	
Dextrose	4500.000000
Putrescine 2HC1	0.053700
Sodium Pyruvate	81.666700
Ascorbic Acid	17.333300
Biotin	0.202400
D-Calcium Pantothenate	0.160000
Sodium Pantothenate	0.337330

Choline Chloride	5.486700
Folic Acid	1.100000
i-Inositol	7.333300
Nicotinamide	0.679000
Na2 alpha Tocopherol PO4	0.003300
Glutathione (Reduced)	0.016700
Menadione Na Bisulfite	0.003300
Pyridoxine HCl	0.020700
Pyridoxal HCl	0.666700
Riboflavin	0.079300
Thiamine HCl	0.780000
Vitamin B12	0.973300
Calciferol	0.033300
Methyl Linoleate	0.010000
Vitamin A Acetate	0.033000
Linoleic Acid	0.028000
Lipoic Acid	0.136700

Preparation of Basal Medium - for a final volume of **100L**

Ninety liters of deionized-distilled water is measured into an appropriate mixing vessel. One 100L packet of ball-mill ground powdered media (see above) is added. The pH of the medium is adjusted to 7.2 using 1N HCl. The volume of the medium is brought to 100L by the addition of water. The medium may then be sterilized by membrane filtration using a 0.2 micron cellulose acetate filter.

Example 2 Preparation of Medium MR1-3

Medium MR1-3 contains the basal medium of Example 1 supplemented with 5,000 mg/l TC Yeastolate (Difco, Detroit, Michigan), 500 mg/l bovine serum albumin (BSA) (Armour, Kankakee, Illinois) 10 mg/l bovine insulin (Waitaki, Toronto, Canada), 10 mg/l bovine transferrin (Sigma Chemical Co., St. Louis, Missouri), 0.12 mg/l oleic acid (Ameresco, Cleveland, Ohio), 0.20 mg/l linoleic acid (Ameresco), 0.028 mg/l linolenic acid (Ameresco), 2 mg/l cholesterol (Ameresco), 66.67 mg/l anhydrous calcium chloride, and 24 mg/l anhydrous magnesium sulfate. The medium is prepared as follows:

For a final volume of **100L**

1. Measure 90 liters of deionized-distilled water into an appropriate mixing vessel.
2. Add one 100L packet of ball-mill ground powdered media (from Example 1).
3. Add 2.4 grams of $MgSO_4$ (anhydrous) and mix until dissolved.
4. Add 6.7 grams of $CaCl_2$ (anhydrous) and mix until

dissolved.
5. Add 500 grams of TC Yeastolate, mix until dissolved.
6. Add 50 grams of BSA, mix until dissolved.
7. Add 220 grams of $NaHCO_3$, mix until dissolved.
8. Add 1 gram of insulin, 1 gram of transferrin (or 100 ml of ferric fructose) and mix until dissolved.
9. Dissolve 12 mg of Oleic acid, 20 mg of Linoleic acid, 2.8 mg of Linolenic acid, and 200 mg of cholesterol in 100 mls of absolute ethanol, and add this fatty acid mix to the mixing vessel.
10. Adjust the pH to 7.2 using 1N HCl.
11. Bring the volume to 100 liters and mix thoroughly.
12. Filter sterilize using a 0.2 micron cellulose acetate filter.
13. Check osmolarity and record.
14. Store at 4°C for up to six months.

Example 3 Preparation of Medium MR1-6

Medium MR1-6 is contains the basal medium of Example 1 supplemented with 5,000 mg/l TC Yeastolate (Difco, Detroit, MIchigan), 500 mg/l bovine serum albumin (Armour, Kankakee, Illinois), 10 mg/l bovine insulin (Waitaki, Toronto, Canada), 10 mg/l bovine transferrin (Sigma Chemical Co., St. Louis, Missouri), 0.12 mg/l oleic acid (Ameresco, Cleveland, Ohio), 0.20 mg/l linoleic acid (Ameresco), 0.028 mg/l linolenic acid (Ameresco), and 2 mg/l cholesterol (Ameresco). The medium is prepared in the same way as medium MR1-3 in Example 2 except that steps 3 and 4 are omitted. In this medium no additional $MgSO_4$ or $CaCl_2$ is added.

Example 4 Preparation of Medium MR1-7.

Medium MR1-7 contains the basal medium of Example 1 supplemented with 5,000 mg/l TC Yeastolate (Difco, Detroit, Michigan), 1,000 mg/l Dextran T-500 (Pharmacia, Piscataway, New Jersey), 10 mg/l bovine insulin (Waitaki, Toronto, Canada), 10 mg/l bovine transferrin (Sigma Chemical Co, St. Louis, Missouri), 0.12 mg/l oleic acid (Ameresco, Cleveland, Ohio), 0.20 mg/l linoleic acid (Ameresco), 0.028 mg/l linolenic acid (Ameresco), and 2 mg/l cholesterol (Ameresco). Mediun MR1-7 is prepared in the same way as medium MR1-3 in Example 2 except that steps 3 and 4 are omitted and Dextran T-500 replaces bovine serum albumin in step 6. At step 6, 100 grams of Dextran T-500 are added and mixed until dissolved.

Example 5 Cell Culture

CHO cells transformed to produce soluble T4, a soluble form of the T-4 lymphocytic cell receptor (cell line 37-80N), were cultured in four different media: serum containing medium Alpha (-) MEM/5% Fetal bovine serum (FBS), and the media described in Examples 2, 3, and 4. 5×10^5 cells per milliliter were cultured for 7 days after seeding in 250 ml SP flasks with 150 ml of medium. Total cell number was determined by Coulter counter, and viability was determined by trypan blue dye exclusion using a hemocytometer. Concentration of ST4 was determined by an ELISA-based assay. At day two after seeding, the serum-free media showed greater number of cells than the serum containing medium. In serum-containing medium, there were approximately 1.3×10^6 cells, whereas in the serum-free media there were approximately 1.6×10^6 cells. At days 3 through 7 significantly more cells were present in the serum-free media than the serum containing medium. At day 3, there were approximately 2.4×10^6 cells in the serum-containing medium and approximately 3.3×10^6 cells in the serum-free media. At day 4, the total number of cells in the serum-containing medium had dropped slightly to about 2.25×10^6 cells. In contrast, the number of cells in the serum-free media had increased to approximately 3.6×10^6 cells in MR1-7, 4.1×10^6 cells in MR1-3, and 4.3×10^6 cells in MR1-6. By day 7, the total number of cells in medium MR1-7 had increased to approximately 4.0×10^6 cell, and the number of cels in the other media remained at levels comparable to the levels at day 4.

By three days post seeding, cells grown in the serum-free media produced significantly more sT4 than did cells grown in the serum containing medium. The difference in amount of sT4 product became more pronounced at days 4-7. At day 7, cells cultured in the serum free media produced from about 75 to 87 micrograms of sT4 per milliliter of medium, whereas cells cultured in the serum containing medium produced about 35 micrograms of sT4 per milliliter of medium.

Claims

1. A serum-free mammalian cell culture medium comprising:

(a) a synthetic basal medium designed for mammalian cell culture;

(b) about 0.1 to about 10 grams per liter hydrolyzed yeast;

(c) about 0.1 to about 5 grams per liter of dextran or albumin;

(d) about 2 to about 20 milligrams per liter insulin;

(e) 0 to about 100 milligrams per liter of a compound selected from the group consisting of transferrin, ferric fructose, ferrous citrate and ferrous sulfate; and

(f) a fatty acid component consisting of oleic acid, linoleic acid and linolenic acid in a ratio of about 0.6 : 1 : 0.14 milligrams of fatty acid per liter.

2. The serum free mammalian cell culture medium of claim 1 further comprising 0 to about 10 milligrams per liter cholesterol.

3. The serum-free mammalian cell culture medium of claim 1 further comprising 0 to about 200 milligrams per liter anhydrous calcium chloride and 0 to about 100 milligrams per liter anhydrous magnesium sulfate.

4. The medium of claim 1 wherein said hydrolyzed yeast is present in the medium in the amount of about five grams per liter.

5. The medium of claim 1 wherein albumin is present in said medium in the amount of about 0.5 grams per liter.

6. The medium of claim 5 wherein said albumin is bovine serum albumin.

7. The medium of claim 1 wherein said dextran is present in said medium in the amount of about one gram per liter.

8. The medium of claim 7 wherein said dextran is dextran having a molecular weight of about 500,000.

9. The medium of claim 1 wherein said insulin is present in said medium in the amount of about 10 milligrams per liter.

10. The medium of claim 1 wherein transferrin is present in

the amount of about 10 milligrams per liter.

11. The medium of claim 1 wherein oleic acid is present in the amount of about 0.12 milligrams per liter; linoleic acid is present in the amount of about 0.20 milligrams per liter; and linolenic acid is present in the amount of about 0.028 milligrams per liter.

12. The medium of claim 2 wherein cholesterol is present in the amount of about two milligrams per liter.

13. The medium of claim 3 wherein said calcium chloride is present in the amount of about 66 to about 67 milligrams per liter; and magnesium sulfate is present in the amount of about 24 milligrams per liter.

14. A serum-free mammalian cell culture medium comprising:

(a) a synthetic basal medium designed for mammalian cell culture;

(b) about 5 grams per liter hydrolyzed yeast;

(c) about 1 gram per liter of albumin;

(d) about 10 milligrams per liter insulin;

(e) about 10 milligrams per milliliter transferrin;

(f) a fatty acid component consisting of about 0.12 milligrams per liter oleic acid, about 0.20 milligrams per liter linoleic acid and about 0.028 milligrams per liter linolenic acid; and

(g) about 2 milligrams per liter cholesterol;

15. The medium of claim 14 further comprising

about 66 to about 67 milligrams per liter anhydrous calcium chloride; and

about 24 milligrams per liter anhydrous magnesium sulfate.

16. A serum-free mammalian cell culture medium comprising:

(a) a synthetic basal medium designed for mammalian cell culture;

(b) about 5 grams per liter hydrolyzed yeast;

(c) about 1 gram per liter dextran having a molecular weight of about 500,000;

(d) about 10 milligrams per liter insulin;

(e) about 10 milligrams per liter transferrin;

(f) a fatty acid component consisting of about 0.12

10 milligrams per liter oleic acid, about 0.20 milligrams per liter linoleic acid and about 0.028 milligrams per liter linolenic acid; and

(g) about 2 milligrams per liter cholesterol.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06837

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00
U.S.Cl.: 435/240.1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	435/3; 435/31; 435/240.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,Y	US, A, 5,024,947(INLOW) 18 June 1991, see entire document.	1,5-10,14,16
Y,E	US, A, 5,063,157(STOCKINGER) 05 November 1991, see entire document.	1,2,4,11,12
Y	In Vitro Cellular & Developmental Biology, Volume 21, No. 10, issued October 1985, F. Gasser et al, "Long-Term Multiplication of the Chinese Hamster Ovary (CHO) Cell Line in a Serum-Free Medium", pages 588-592, see entire document.	1,9,10,14,16
Y	US, A, 4,786,599(CHESSEBEUF ET AL) 22 November 1988, see entire document.	1,5,6,7,8,11, 14,16
Y	Cell, Volume 22, issued December 1980, D. Barnes et al, "Serum-Free Cell Culture: A Unifying Approach," pages 649-655, see entire document.	1-16
Y	Analytical Biochemistry, Volume 102, issued 1980,Barnes et al,"Methods for Growth of Cultured Cells in Serum-Free Medium," pages 255-270, see entire document.	1-16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

30 December 1991

Date of Mailing of this International Search Report

21 FEB 1992

International Searching Authority

ISA/US

Signature of Authorized Officer

Jane Williams

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Biotechnology, Volumn 6, issued December 1988, B. Maiarella et al, "Large-Scale Insect Cell-Culture for Recombinant Protein Production", pages 1406-1410 see entire document.	1,2,4,11,12, 14,16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.